

## NOTE

# $\beta$ (1-3)Glucanoyltransferase Gel4p Is Essential for *Aspergillus fumigatus*<sup>▽</sup>

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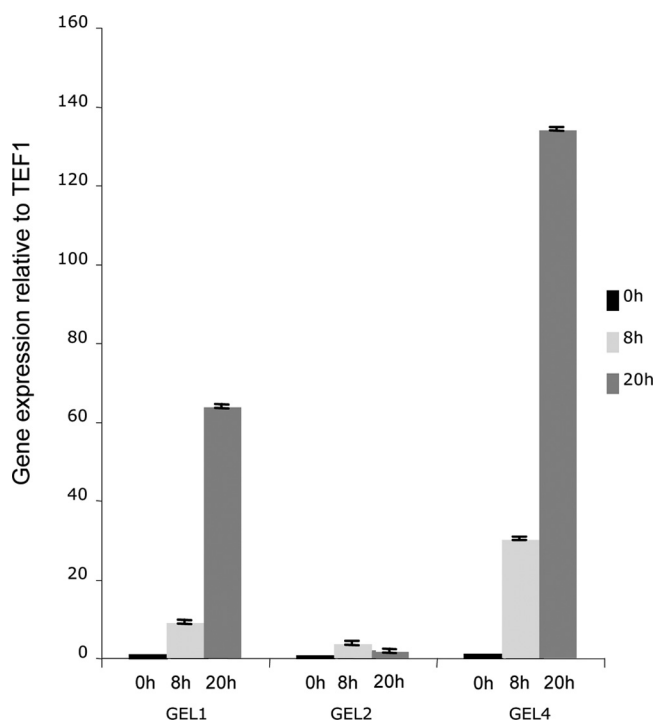
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**The  $\beta$ (1-3)glucanoyltransferase GEL family of *Aspergillus fumigatus* contains 7 genes, among which only 3 are expressed during mycelial growth. The role of the GEL4 gene was investigated in this study. Like the other Gels, it encodes a glycosylphosphatidylinositol (GPI)-anchored protein. In contrast to the other  $\beta$ (1-3)glucanoyltransferases analyzed to date, it is essential for this fungal species.**

$\beta$ (1-3)Glucan is the main component of the fungal cell wall (11). In fungi,  $\beta$ (1-3)glucans are synthesized by a plasma membrane-bound glucan synthase complex. Neosynthesized glucans are then extruded into the periplasmic space (2, 3, 9), where they become branched and covalently linked to other cell wall components, resulting in the formation of three-dimensional rigid structures. In the search of transglycosidase in the filamentous fungus *Aspergillus fumigatus*,  $\beta$ (1-3)glucanoyltransferases were identified and classified as a unique family (GH72) in the Carbohydrate-Active enZYmes database (<http://www.cazy.org/>). These enzymes cleave the  $\beta$ (1-3) bond of a  $\beta$ (1-3)glucan oligosaccharide with at least 10 glucose units and transfer the newly formed reducing end (>5 glucose units) to the nonreducing end of another  $\beta$ (1-3)glucan oligosaccharide, resulting in the elongation of the  $\beta$ (1-3)glucans. This reaction can proceed *in vitro* until the neosynthesized  $\beta$ (1-3)glucan becomes insoluble. Initially demonstrated biochemically, the requirement for long-chain  $\beta$ (1-3)glucan oligosaccharide has now been confirmed by the analysis of the first crystal structure obtained in this transglycosidase family (7, 8). First discovered in *Aspergillus fumigatus* and named Gelp for glucan elongase, this activity has been found in all fungal species investigated to date and could be assigned to orthologous proteins, such as Gasp or Phrp, that were known to be involved in cell wall integrity but were endowed with an unknown biochemical function (12, 13, 14).

**Three members of the *A. fumigatus* GEL family are expressed during mycelial growth.** In *A. fumigatus*, the GEL family contained 7 open reading frames (ORFs) named GEL1 (AFUA\_2G01170), GEL2 (AFUA\_6G11390), GEL3 (AFUA\_2G12850), GEL4 (AFUA\_2G05340), GEL5 (AFUA\_8G02130), GEL6 (AFUA\_3G13200), and GEL7 (AFUA\_6G12410). Gel2p to Gel7p showed 37, 24, 28, 26, 29, and 26% identity with *A. fumigatus* Gel1p (AfGel1p), respectively. Among them, only GEL1, GEL2, and GEL4

were expressed constitutively during mycelial growth. Quantitative real-time PCR (qRT-PCR) assays using RNA extracted at different times of culture and amplified as described previously (10) showed that among those 3 genes,



**FIG. 1.** Expression level of the GEL1, GEL2, and GEL4 genes during growth. Gene expression was determined by real-time RT-PCR in resting conidia (0 h), germinated conidia (8 h), and mycelia (20 h) incubated in 3% glucose-1% yeast extract at 37°C. Triplicate biological RT-PCR assays were performed using 1× iQ SYBR green master mix, and expression levels were normalized to those of TEF1. The threshold cycle ( $2^{-\Delta\Delta CT}$ ) method was used to determine fold changes of expression. Primer pairs used were Gel1a (TTCGCTACCGTTGATGCTTT CG)-Gel1b (TGCGGCTACGGATGTACTGAC); Gel2a (GCCTCT CCGACGCTAACAC)-Gel2b (GGTATTGGACTCGCCGCTAG); Gel4a (ATACGCCACCGACGAGGAC)-Gel4b (GGAAGAATCAC CGCACCACTC); and Tef1a (CCATGTGTGTGCGAGTCCTTC)-Tef1b (GAACGTACAGCAACAGTCTGG).

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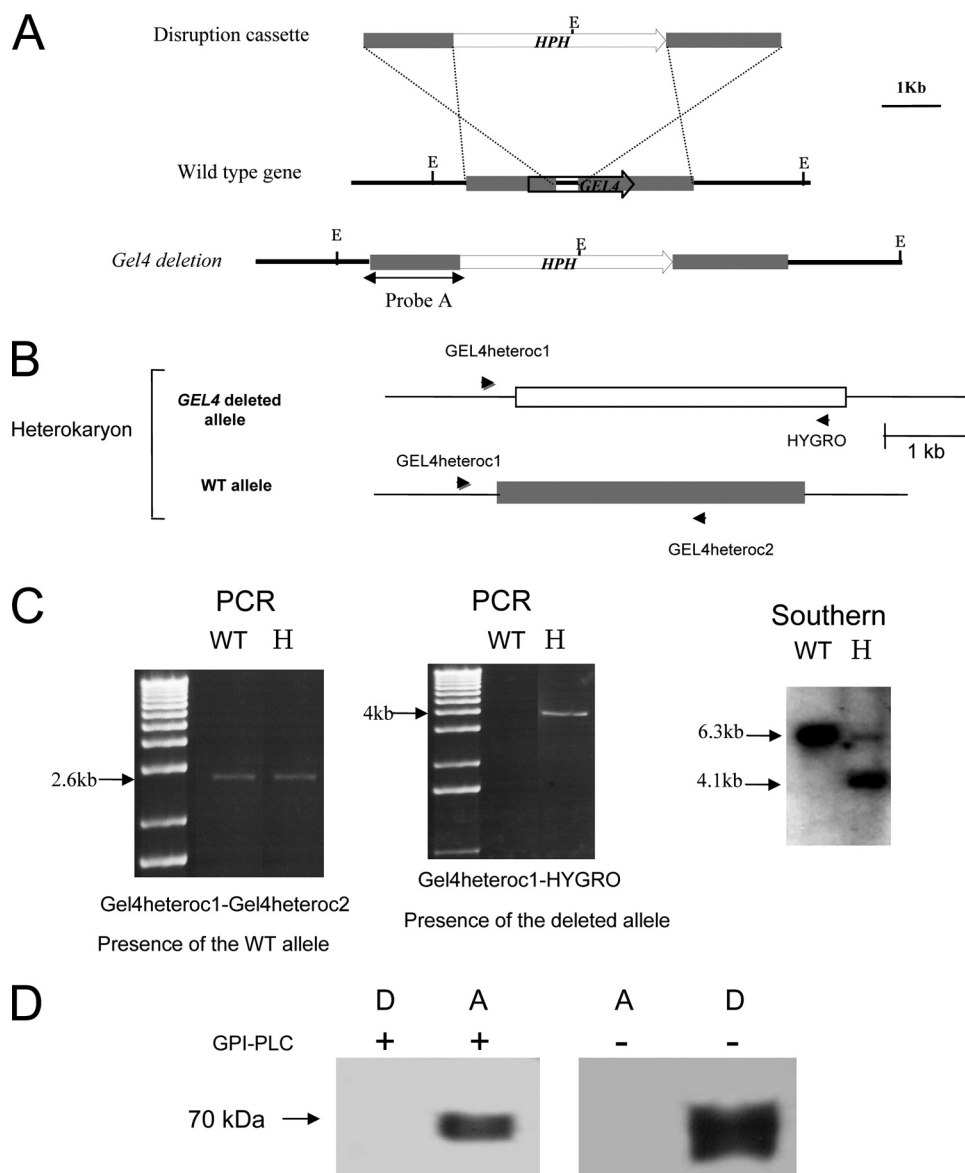


FIG. 2. (A to C) Deletion of *GEL4* in *A. fumigatus* using the heterokaryon rescue method. (A) Strategy used to insert the hygromycin resistance gene (*HPH*) inside *GEL4* (*E*, *EcoRI* site). (B and C) PCR and Southern blot analyses showing the presence of both wild-type and mutated alleles in the heterokaryon (H) and the absence of the mutated allele in the wild-type strain (WT). The PCR primer pair used in the heterokaryon and in the wild-type strain to show the presence of a wild-type allele is *GEL4*heteroc1 (CCAAACCAAATCATCAGCCCCAGCCCCAATC)-*GEL4*heteroc2 (GTAGTCGGCCAAGTCGGCACGGATGTGGGCG), and the pair used to show the presence of a deleted allele is *GEL4*heteroc1-HYGRO (CGACAGCGTCTCCGACCTGATGCAGCTCTC). For Southern blotting, genomic DNA was digested with *EcoRI* and hybridized with probe A. DNA isolated from the wild-type strain showed one band of 6.3 kb corresponding to a copy of the wild-type allele, whereas the DNA isolated from the heterokaryon showed the wild-type allele and the mutated allele at 4.1 kb (see panel A). (D) Solubilization of membrane-bound Gel4p with GPI-PLC. Aqueous upper phase (marked "A") and detergent lower phase (marked "D") after Triton X-114 partitioning of GPI-PLC-treated (+) or control (-) membranes; immunolabeling with anti-Gel4p hyperimmune antiserum.

*GEL4* was the most expressed at all times of growth (Fig. 1). *GEL1* and *GEL2* were previously characterized. Disruption of *GEL1* did not result in a phenotype, whereas a *gel2* mutant and the double mutant *gel1 gel2* exhibited slower growth, associated with a decrease of  $\beta(1-3)$ glucan content, an increase of chitin, and abnormal conidiogenesis (14). The function of *GEL4* is reported here.

***GEL4* is an essential gene in *A. fumigatus*.** To delete *GEL4*, a deletion cassette containing the hygromycin resistance

marker inserted inside the *GEL4* ORF was constructed (Fig. 2A). The resulting fusion PCR product was used for transformation of protoplasts of the *A. fumigatus* *akuB*<sup>ΔKu80</sup> strain (4). In spite of repeated attempts, no mutant was recovered by classical gene replacement experiments, suggesting that this gene was essential. The heterokaryon rescue technique recently used to demonstrate gene essentiality in *A. fumigatus* (10, 15) was applied here. The presence of both wild-type and mutant alleles in heterokaryons was

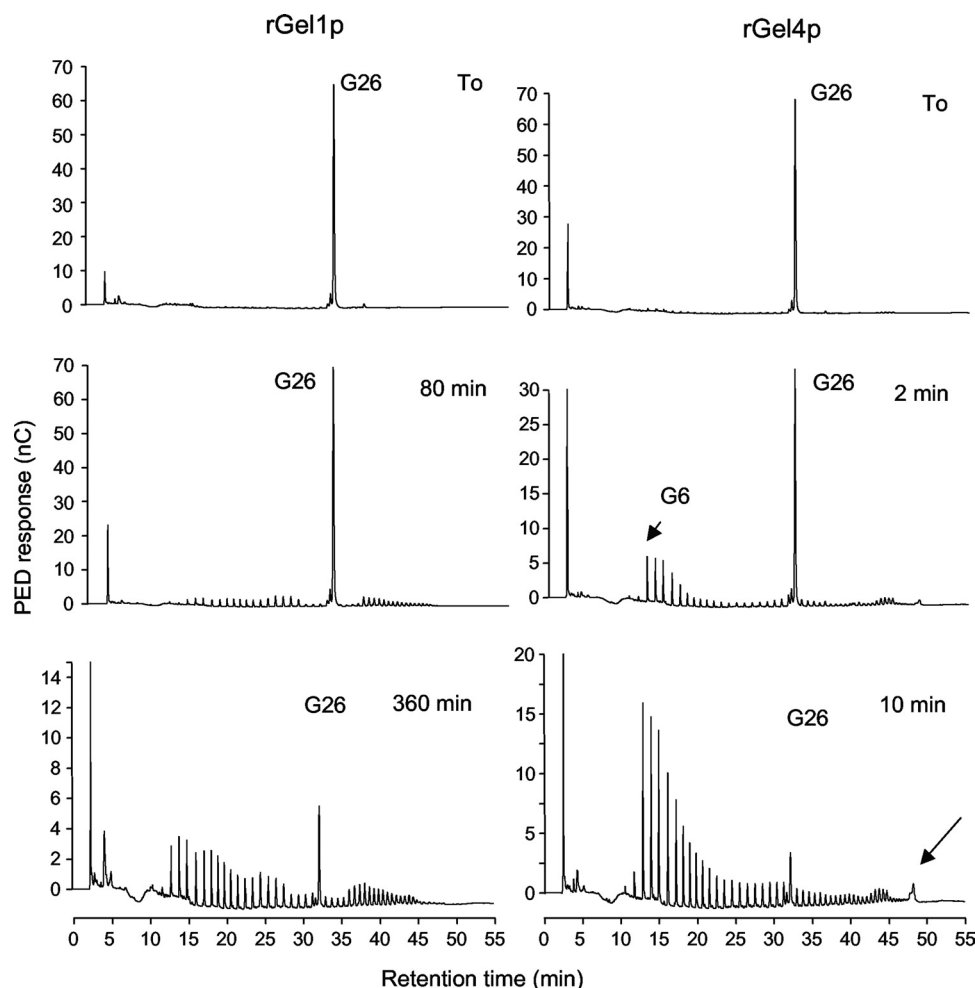


FIG. 3. HPAEC analysis of products obtained from the incubation of the recombinant Gel1p and Gel4p with reduced laminarioligosaccharides. Recombinant proteins (1.17  $\mu$ g purified) were incubated with 9 mM reduced laminarioligosaccharide containing 26 glucose units in 28  $\mu$ l of 50 mM sodium acetate buffer, pH 5.5, at 37°C. Aliquots (1.5  $\mu$ l) supplemented with 200  $\mu$ l 50 mM NaOH were analyzed by HPAEC with a CarboPac PA200 column (3 by 250 mm; Dionex) and a pulsed electrochemical detector. Note that the last peak (indicated by an arrow) corresponds to water-insoluble products.

shown by PCR and Southern blotting, the primer pair GEL4heteroc1-GEL4heteroc2 amplified a DNA fragment corresponding to the wild-type allele, and the amplification obtained with primers GEL4heteroc1-HYGRO indicated the presence of the *GEL4*-deleted allele (Fig. 2B and C). Uninucleate conidia isolated from this heterokaryon were unable to germinate and grow on selective medium, even in the presence of 1.2 M sorbitol, indicating that *GEL4* was essential for *A. fumigatus*.

**Gel4p is a GPI-anchored protein.** In membrane preparations, Gel4p with an apparent  $M_r$  of 70 was detected with an antiserum obtained from a rabbit immunized with the peptide C<sub>445</sub>DFDGKAQTKKGADA<sub>460</sub> of Gel4p conjugated to key-hole limpet hemocyanin (KLH). A membrane fraction was treated with phosphatidylinositol (PI)-phospholipase C (PLC) of *Bacillus thuringiensis* (able to cleave the glycosylphosphatidylinositol [GPI] anchor) and submitted to a partitioning with Triton X-114, as described previously (12). Following this treatment, Gel4p was present in the aqueous phase, whereas it remained in the detergent phase in the absence of PI-PLC

treatment (Fig. 2D). These data showed that Gel4p was bound to the membrane through a GPI anchor.

**Gel4p has  $\beta$ (1-3)glucanosyltransferase activity.** The sequence similarities of Gel4p with Gel1p and Gel2p, including the conservation of the two aspartic acid residues essential for the transglycosidase activity, FF(A/S)GNEV (E is the acid-base donor) and F(F/L)SE(Y/F)GCN (E is the nucleophilic residue) (13), suggested that Gel4p had  $\beta$ (1-3)glucanosyltransferase activity typical of the GH72 family (5, 13, 17). In order to analyze the enzymatic activity of Gel4p, a recombinant Gel4p (rGel4p; encompassing amino acids 20 to 488) was produced in *Pichia pastoris* by following a strategy used previously to produce rGel1p and rGel2p (14). Gel4p produced in *P. pastoris* had an apparent  $M_r$  of 70 and was glycosylated, as shown by a reduction in  $M_r$  to 52 after peptide *N*-glycosidase F (PNGase F) treatment (data not shown). The recombinant Gel4p was incubated with reduced laminarioligosaccharides of different sizes, and the products of the reaction were analyzed using a Dionex high-performance anion-exchange chromatography with pulsed electrochemical detection (HPAEC-PED)

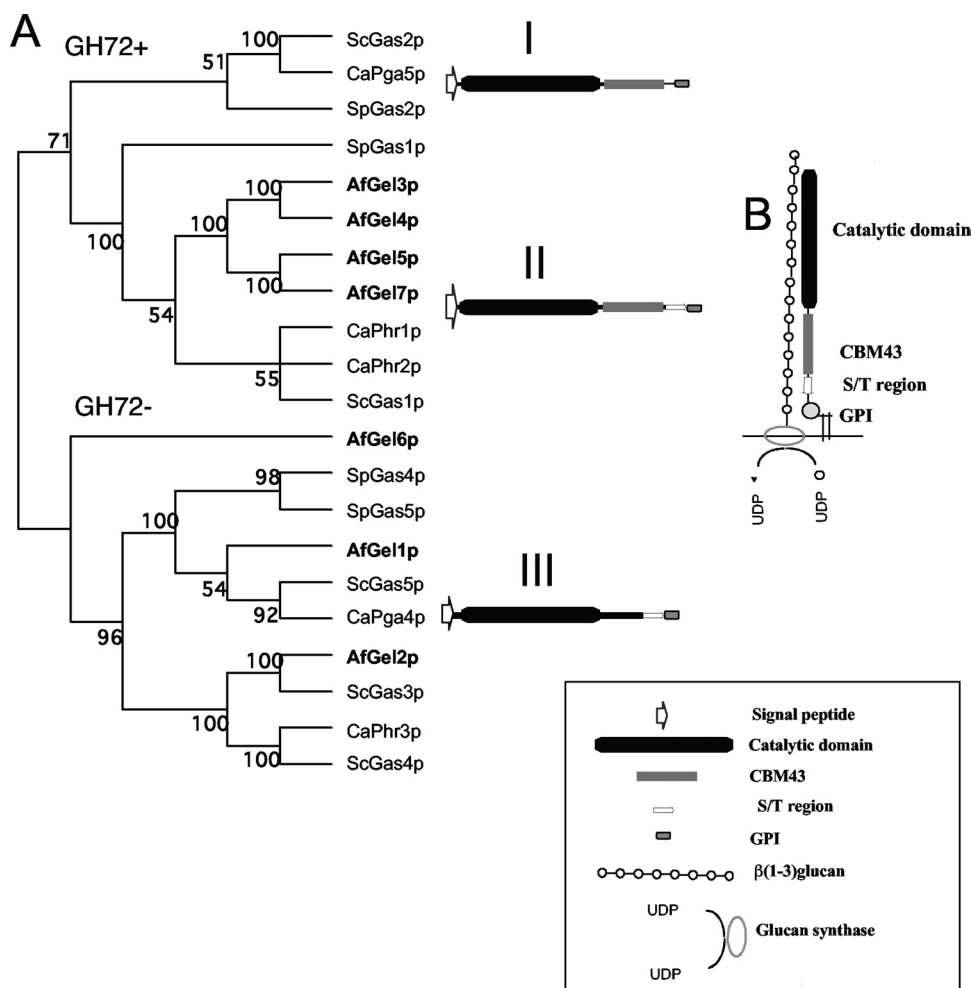


FIG. 4. (A) Phylogenetic tree of proteins of *A. fumigatus*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Candida albicans* belonging to the GH72 family. Protein alignment was made with ClustalX, bootstrap values were calculated with PhyML, and the tree was drawn with MEGA software. The protein sequences used are from the indicated species, as follows (Swiss-Prot accession numbers are listed in parentheses): from *S. pombe*, Spgas1p (Q9P378), Spgas2p (Q9USU5), Spgas4p (Q9Y7Y7), and Spgas5p (O13692); from *S. cerevisiae*, Gas1p (P22146), Gas2p (Q06135), Gas3p (Q03655), Gas4p (Q08271), and Gas5p (Q08193); from *Candida albicans*, CaPhr1p (P43076), CaPhr2p (O13318), CaPhr3p (Q9P8R2), CaPga4p (Ca019.4035), and CaPga5p (Ca019.3693); and from *Aspergillus fumigatus*, AfGel1p (AFUA\_2G01170), AfGel2p (AFUA\_6G11390), AfGel3p (AFUA\_2G12850), AfGel4p (AFUA\_2G05340), AfGel5p (AFUA\_8G02130), AfGel6p (AFUA\_3G13200), and AfGel7p (AFUA\_6G12410). (Right) Schematized domains of the 3 subfamilies of GH72, consisting of the signal peptide, catalytic domain, cysteine domain or CBM43 (I), S/T-rich domain, and GPI anchor. *Aspergillus fumigatus* proteins are shown in boldface. (B) Hypothetical representation of the *in situ* localization of a member of the GH72<sup>+</sup> ST<sup>+</sup> subfamily, such as AfGel4p. CBM43 directs the catalytic site toward the  $\beta(1-3)$ glucan chain. After being anchored, the catalytic domain is able to modify the oligosaccharide.

system with a CarboPac PA200 column, as described previously (14). As an example, Fig. 3 shows the results of the incubation of rGel4p with an oligosaccharide of 26 glucose residues ( $G_{26r}$ ). The major products obtained were in agreement with those obtained using the previously described two-step reaction scheme (7), as follows: the enzyme acted first as an endoglucanase, released short laminarioligosaccharides, and transferred the new reducing ends formed to another oligosaccharide, resulting in the elongation of the  $\beta(1-3)$ glucan chain, until it became water insoluble (corresponding with the largest peak of the HPAEC profile shown in Fig. 3). Transfer activity was seen with other oligosaccharides of lower degrees of polymerization (rG11, rG13, or rG14). Degradation of the reaction products by an endo( $\beta(1-3)$ )glucanase (as described in reference 6) resulted in the release of glucose and laminar-

ibiose, without that of branched oligosaccharide, confirming that the transfer product contained only linear  $\beta(1-3)$ glucan chains. The analysis comparing rGel4p to rGel1p under the same experimental conditions showed that rGel4p had a higher avidity for the  $\beta$ -glucan than rGel1p (Fig. 3).

A phylogenetic tree of the GH72  $\beta(1-3)$ glucanotransferase family showed that its members can be grouped into 3 subfamilies based on the presence or absence of CBM43 (1), a carbohydrate-binding module most commonly associated with  $\beta(1-3)$ glucan binding activity and a serine/threonine-rich region that contains O-glycosylation putative sites (Fig. 4A). These two domains are present only in the members of the family that have the most important morphogenetic role (16, 17; M. De Medona Redondo and C. Vazquez de Aldana, personal communication). Although the pres-

ence of these domains are not important for  $\beta(1-3)$ glucanoyltransferase activity (17), they may be essential to localize the proteins at the right places for its morphogenetic activity: O-glycosylated stalks elevate the  $\beta(1,3)$ glucan binding domain above the plasma membrane, where the protein is anchored with GPI, and place the catalytic site in the best position to act on the nascent  $\beta(1,3)$ glucan (Fig. 4B).

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